***Int J Pharm, 513*(1-2), 404-409**. doi:10.1016/j.ijpharm.2016.09.053

Alginate nanoparticles protect ferrous from oxidation: potential iron delivery system

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***Int J Pharm, 513*(1-2), 404-409**. doi:10.1016/j.ijpharm.2016.09.053

Abstract

A novel, efficient delivery system for iron (Fe^{2+}) was developed using the alginate biopolymer. Iron loaded alginate nanoparticles were synthesized by a controlled ionic gelation method and was characterized with respect to particle size, zeta potential, morphology and encapsulation efficiency. Successful loading was confirmed with Fourier Transform Infrared spectroscopy and Thermogravimetric Analysis. Electron energy loss spectroscopy study corroborated the loading of ferrous into the alginate nanoparticles. Iron encapsulation (70%) was optimized at 0.06% Fe (w/v) leading to the formation of iron loaded alginate nanoparticles with a size range of 15-30 nm and with a negative zeta potential (-38 mV). The *in vitro* release studies showed a prolonged release profile for 96 h. Release of iron was around 65-70% at pH of 6 and 7.4 whereas it was less than 20% at pH 2. The initial burst release upto 8 h followed zero order kinetics at all three pH values. All the release profiles beyond 8 h best fitted the Korsmeyer-Peppas model of diffusion. Non Fickian diffusion was observed at pH 6 and 7.4 while at pH 2 Fickian diffusion was observed.

Keywords: Anemia, ferrous sulfate, alginate nanoparticles, iron loaded nanocomposite, bioavailability

Chemical compounds studied in this article

Sodium alginate (PubChem CID: 5102882); Ferrous sulphate (PubChem CID: 24393); Calcium chloride (PubChem CID: 5284359; Sorbitan monooleate (PubChem CID: 9920342); L-Ascorbic acid (PubChem CID: 54670067)

1. Introduction

Iron deficiency has become the most common nutritional deficiency today (Horton & Ross, 2003).Since the body requires iron to synthesize its oxygen transport proteins in particular, hemoglobin and myoglobin (Abbaspour, et al., 2014), iron deficiency often leads to anemia. Iron deficiency results from insufficient intake of iron from diet and poor utilization of iron from ingested food or a combination of both factors (Gaucheron, 2000). The only proven way to lessen this issue is to increase iron intake, either by providing medicinal iron (supplementation) or by adding iron into the diet (fortification), basically to food staples (such as wheat and maize flour) or condiments (such as soy sauce, fish sauce, sugar and salt)(Cook & Reusser, 1983). Both these approaches heavily utilize ferrous based products (Patil, et al., 2012). In spite of the development of newer oral iron preparations, ferrous sulphate still remains the first line of treatment (Martínez-Navarrete, et al., 2002) mainly due to its low cost and high availability (Patil, et al., 2012). However, the ferrous ion based supplements are not always compliant due to adverse gastrointestinal effects such as nausea, vomiting and gastric distress (Saha, et al., 2007), hence limiting the benefits of the iron supplementation therapy. In addition, some severe conditions may occur, such as allergic reactions, black and tarry stools, fever and continuing stomach pain (Hosny, et al., 2015). Further, its variability is very high in iron absorption, thus effecting the bioavailability (Bregman , et al., 2013).

Therefore, there is a need for developing a novel, stable system which is able to increase the iron bioavailability. Previous work on encapsulation techniques for the treatment of anemia with nano drug delivery systems reports the use of ferrous sulphate loaded solid lipid nanoparticles (Zariwala, et al., 2013), (Hosny, et al., 2015). The prevention of exposure of ferrous directly to the gastrointestinal tract and the slow release property would be advantageous during its oral uptake. Furthermore, the need for high doses of iron to obtain the therapeutic effect can be avoided through slow release, minimizing the side effects associated with conventional oral iron supplements.
Encapsulation protects ferrous ions interacting with other materials and prevents the direct contact of ferrous with gastrointestinal lumen, thus reducing the possible adverse effects (Hosny, et al., 2015), (Xia & Xu, 2005). Alginate biopolymer system has promising properties (Sosnik 2014) and is safe to be used as an oral carrier for iron. Alginic acid and sodium alginate have turned out to be the most extensively explored mucoadhesive biomaterial with good cytocompatibility and

biocompatibility (Lee & Mooney, 2012) (Sarei, et al., 2013). One major advantage of using alginate in oral delivery formulations is their property of being in solid like structure at gastric conditions due to the formation of alginic acid. Hence, it protects the encapsulant inside the core (Draget & Taylor, 2011). Also, alginate beads dissolve under neutral and basic pH values which is more effective in iron delivery since the absorption of iron occurs mainly in the duodenum in which the pH is around 7.0-8.5 (Draget & Taylor, 2011).

In the current study, we have examined the potential of alginate nanoparticles for oral iron delivery. The aim of our study was to formulate alginate nanoparticles to encapsulate ferrous sulphate and evaluate the release kinetics of iron in pH varying buffer solutions with a view to examining its potential as a nano-biopolymeric carrier of Fe^{2+}.

2. Materials and Methods

2.1 Materials

Low viscosity sodium alginate, ferrous sulphate, calcium chloride, Sorbitan monooleate (span 80) and L-ascorbic acid were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). All other reagents were of analytical grade and used directly. Snake skin dialysis tubing (MWCO 3500) was purchased from Thermo Scientific USA.

2.2 Preparation of ferrous loaded alginate nanoparticles

The nanoparticles were prepared by ionic gelation based on methods described previously (Daemi & Barikani, 2012). The pH of a 0.3% w/v solution of sodium alginate (40.0 mL) was adjusted to around 5 and then stirred with span 80 for 2 h at 60 ^{o}C to obtain a homogeneous mixture. This sodium alginate solution was stirred with 50.0 mL of ferrous sulphate solution in the presence of ascorbic acid where the ratio of ferrous sulphate to ascorbic acid was always 15:1 and with varying iron concentrations (1%, 2% and 3%, w/w alginate). The above complex was gelated by drop wise addition of 40.0 mL of $CaCl_2$ solution (0.1% w/v) while stirring at a high speed for 1 h. The nanoparticle suspension was refrigerated overnight and centrifuged at 9000 rpm for 45 minutes to obtain the nanoparticle pellet.

2.3 *Characterization of ferrous loaded alginate nanoparticles*

2.3.1 particle size and zeta potential measurements

The average particle size and size polydispersity of the nanoparticles dispersed in distilled water were determined by dynamic light scattering technique at 25 ^{o}C using a particle size analyzer (Zetasizer Nano ZS, Malvern Instruments, UK) at a fixed scattering angle of 90^{o}. The zeta potential of nanoparticles was measured using the Zeta potential analyzer (Zetasizer Nano ZS, Malvern Instruments, UK). All measurements were performed in triplicate.

2.3.2 Fourier Transform Infrared spectroscopy (FTIR) characterization and thermal analysis

FTIR spectra of sodium alginate, alginate nanoparticles and iron loaded alginate nanoparticles were obtained with a Bruker Vertex 80 IR spectrometer (Germany) at a resolution of 4 cm^{-1} from 4000 to 400 cm^{-1}. Thermal decomposition of sodium alginate, alginate nanoparticles and ferrous loaded alginate nanoparticles were analyzed using a SDT Q600 thermogravimetric analyzer (TA Instruments, USA) from 25 ^{o}C to 800 ^{o}C using a ramp rate of 10 ^{o}C/ min in air.

2.3.3 Transmission Electron Microscopic (TEM) imaging

A drop of ferrous loaded alginate nanoparticle dispersion was placed on a holey carbon Cu grid and allowed to dry at room temperature. Then nanoparticles were imaged using a high resolution transmission electron microscope (TEM) (JEM 2100, JEOL, Japan) operated at accelerating voltage 200 kV.

2.3.4 Electron energy loss spectroscopy (EELS) study of ferrous loaded nanoparticles

EELS spectrum of ferrous loaded alginate nanoparticles was acquired with an EELS spectrometer (EELS Gatan, Quantum 963, USA) attached to the TEM with the energy resolution of 0.05 eV/channel in STEM spectral imaging mode. EELS spectra of $FeCl_3$ and $FeSO_4$ standards were acquired for a comparison study.

2.4 *Determination of encapsulation efficiency*

The amount of incorporated ferrous in the nanoparticles was determined by thiocyanate colorimetry. The supernatant obtained after centrifugation was subjected to oxidation with 0.15 mol dm^{-3} of $KMnO_4$ in acidic medium to convert all ferrous ions to ferric ions since ferrous ion does not form a coloured complex with thiocyanate. Next, the oxidized supernatant was complexed

with 1 mol dm^{-3} potassium thiocyanate solution and the absorbance was measured at 490 nm using the UV-Visible spectrophotometer (SHIMADZU, UV-3600, UV-VIS-NIR). Then, the concentration was calculated from a calibration plot obtained for ferric ion standard solution. Percentage encapsulation efficiency was calculated as follows.

$$\% \text{ Encapsulation efficiency} = \frac{\text{Amount}_{\text{total}} - \text{Amount}_{\text{supernatant}}}{\text{Amount}_{\text{total}}} \times 100\% \qquad \text{Eq. 1}$$

2.5 *In vitro release study of iron loaded alginate nanoparticles*

The release characteristics of iron from alginate nanoparticles were studied in pH 7.4, 6 and 2 solutions. The iron loaded alginate nanoparticles were dispersed in 5.00 mL of buffer solution and trapped inside a dialysis membrane and this was immersed in 25.00 mL of buffer solution at 37 ºC with mild agitation. Aliquots (3.00 mL) were withdrawn at predetermined time intervals and after the oxidation process with 0.15 mol dm^{-3} of $KMnO_4$ in acidic medium, it was complexed with 1 mol dm^{-3} potassium thiocyanate solution and then the UV absorbance at 490 nm was recorded using the UV-Visible spectrophotometer (SHIMADZU, UV-3600, UV-VIS-NIR). The release medium was refreshed with another 3.00 mL of medium after each withdrawal. All measurements were performed in triplicate. Using the calibration plot the concentrations were calculated hence the cumulative release percentages were determined.

Release profiles obtained for different pH buffer solutions were fitted to 5 different mathematical models used to determine the kinetics of drug release from delivery systems: Zero order, First order, Higuchi, Hixon-Crowell and Korsemeyer-Peppas. The model that exhibited the adjusted R-square closest to unity was selected as the best fit. The functions of the models considered are given below (Singhvi & Singh, 2011)(Lokhandwala, et al., 2013).

Zero order Model

$$Q_t = Q_O + K_O\, t \qquad \text{Eq. 2}$$

Where;

Q_t – amount of drug dissolved in time t

Q_O – initial amount of drug in solution

K_O - zero order release constant

First order Model

$$log\ C_t = \log C_O - kt/2.303 \quad \text{Eq. 3}$$

Where;

C_t - concentration of drug at time t

C_0-initial concentration of drug

k – first order rate constant

Higuchi Model

$$Q_t = k_H t^{1/2} \quad \text{Eq. 4}$$

Where;

Q_t – amount of drug released in time t

k_H – Higuchi dissolution constant

t - time

Hixon-Crowell Model

$$W_O{}^{1/3} - W_t{}^{1/3} = kt \quad \text{Eq. 5}$$

Where;

W_O - initial amount of drug in the pharmaceutical dosage form

W_t - remaining amount of drug at time t

k - constant incorporating surface-volume relation

Korsmeyer-Peppas model

Where;

$$M_t / M_\infty = Kt^n \quad \text{Eq. 6}$$

M_t / M_∞ - fraction of drug released at time t

K - release rate constant

n - release exponent

3. Results and Discussion

3.1 Formation of ferrous loaded alginate nanoparticles

The ionic gelation method was followed to synthesize the ferrous loaded alginate nanocomposite where calcium chloride was used as the cross linker. The preparation included two steps, i.e., droplet formation and droplet solidification. Ferrous chelated alginate droplets formation was achieved by the stirring with span 80 surfactant and then each droplet was solidified by ionic cross linking with calcium ions (Keawchaoon & Yoksan, 2011). The negative charge of sodium alginate is the driving force for complexation of ferrous with the polymer where chelation occurs between the Fe^{2+} with carboxylate and hydroxyl moieties. Around pH 5, the negatively charged carboxylate groups of alginate polymer are electrostatically linked with calcium ions. Since pK_a value of alginate lies between 3.4 to 4.4 (Goswami, et al., 2014), the alginate biopolymer will be present as dissociated carboxylate ions around pH 5 facilitating the efficient crosslinking with calcium ions. Ascorbic acid was used as an antioxidant to protect the ferrous ion against oxidation.

A preliminary experiment was carried out to optimise the iron content in the alginate nanoparticles. Thus formulations were prepared with varying iron concentrations (1%, 2% and 3%, w/w alginate) and iron encapsulation efficiencies were determined. The %encapsulation efficiencies were 75%, 48% and 30% with the iron concentrations 1%, 2% and 3% w/w alginate respectively. Increased iron concentration correlated with a decreased amount of encapsulated iron. The higher ionic strength of ferrous sulphate being a strong electrolyte in water may have an influence in encapsulation with alginate (Xia & Xu, 2005).Thus, the highest encapsulation efficiency (75%), and hence the optimal concentration, was obtained with the formulation where the iron concentration was 1%, w/w with alginate.

3.2 Successful loading of ferrous into alginate nanoparticles

Changes in the FTIR spectrum of alginate nanoparticles on loading with ferrous sulphate indicate successful loading. The spectra of unloaded alginate nanoparticles and ferrous loaded alginate nanoparticles are shown in figure 1. The analysis of spectra indicates the presence of ferrous in alginate nanoparticles.

The –OH stretching of hydroxyl of alginate nanoparticles (3340 cm^{-1}) had shifted to 3350 cm^{-1} in the spectrum of ferrous loaded alginate nanoparticles. The position of carboxylate stretching peaks had also changed upon ferrous loading: the asymmetric $–COO^-$ stretching vibration at 1580 cm^{-1}

of the native alginate nanoparticles had shifted to 1597 cm^{-1} in the spectrum of ferrous loaded alginate nanoparticles. The foregoing indicated the binding of Fe^{2+} with OH and COO^- moieties.

To further evaluate Fe^{2+} loading onto alginate nanoparticles (Fig. 2), thermo gravimetric analysis (TGA) was carried out (Fig. 2). In the unloaded alginate nanoparticles, TGA showed that degradation had occurred in four steps whereas with ferrous loaded alginate nanoparticles had an additional decomposition step from 110-180 ^oC due to the decomposition of ascorbic acid. Specifically, the 30% weight loss at 273 ^oC (the inflection point temperature) is followed by a 25% weight loss at 539 ^oC in the unloaded alginate nanoparticles. However, in the ferrous loaded particles the same weight loss is observed at 267 ^oC and 556 ^oC due to the interaction of ferrous with COO^-/OH groups of the alginate. The residual weight of 8% with unloaded alginate nanoparticles might be due to the ash content and the formation of metal oxides. The increased residual weight (20%) with ferrous loaded alginate nanoparticles is due to the formation of additional oxides of ferrous. Therefore, all the above reflect the successful loading of ferrous into alginate nanoparticles.

To determine the elemental composition, in particular the loading of ferrous, and not ferric, into alginate nanoparticles, EELS technique was used. Fe core loss peak for L_3 and L_2 edges observed from EELS represent the excitation of Fe $2p^{3/2}$and $2p^{1/2}$ electrons (Egerton, 2009). An ionization edge appears at slightly different positions depending on the actual electron structure of a given material (Muller, et al., 1998). This difference in the onset position, also referred to as the chemical shift, in most cases does not exceed 1eV (Potapov & Schryvers, 2004). The EELS spectrum obtained for ferrous loaded alginate nanoparticles prepared in the presence of ascorbic acid clearly showed the characteristic L_3 edge peak for Fe^{2+} with a peak maximum at ~ 714.2 eV which is asymmetric with a slight shoulder on the high energy side (Aken & Liebscher, 2002). This edge structure is very similar to the L_3 edge structure of Fe^{2+} acquired for $FeSO_4$ reference standard which has a peak maximum around 714 eV (Fig.3).While this L_3 edge peak has a shifted peak maximum when it is compared with the L_3 edge structure obtained for the reference standard ($FeSO_4$), it is at a different position from $FeCl_3$ with the peak maximum at 713.2 eV (Fig. 3). Thus, around 1 eV energy shift could be observed with L_3 edge peaks of different oxidation states of iron. Therefore, these results confirm the loading of ferrous into alginate nanoparticles without oxidation to the ferric state.

3.3 Physiochemical properties of ferrous loaded nanoparticles

Table 1: Shape, size and surface charge of ferrous loaded alginate nanoparticles where the iron concentration is 1%, w/w alginate.

System	Average Size/ nm	Zeta potential/ mV	% Encapsulation Efficiency
Alginate nanoparticles	25 ± 9	-36 ± 3	
Ferrous loaded alginate nanoparticles	20 ± 6	-38 ± 4	95 ± 4

The particle morphology was examined through the TEM and is depicted in Fig.4. The spherical shape and smooth surfaces of the nanoparticles with no observed aggregation was clearly visible. Dynamic light scattering (DLS) technique was also used to determine the particle size distribution using Zetasizer Nano ZS. The average size of alginate nanoparticles was around 25 ± 9 nm (Table 1), indicating their suitability as a delivery system for iron, for smaller nanoparticles are better for solubility and bioavailability.

The average zeta potential or the surface charge of the alginate nanoparticles was -36 ± 3 mV and the zeta potential of the ferrous loaded alginate nanoparticles was -38 ± 4 mV. Both values were quite similar. Considering the high surface to volume ratio of the nanoparticles, the negative charge is maintained even after the loading of iron. Another favorable point is that due to the potential of the particles they are sufficiently repelled from each other thus avoiding agglomeration.

3.5 In vitro release studies of ferrous from alginate nanoparticles

The *in vitro* release study of ferrous from ferrous loaded alginate nanoparticles was carried out in different pH media to confirm the ferrous encapsulation, to understand the release mechanism and kinetics and to determine the optimum condition (pH of medium) for releasing ferrous from the nanoparticles. The *in vitro* release profile of ferrous from the alginate nanocomposite in different pH buffer solutions (pH 7.4, 6 and 2) are shown in Figure 5. At pH 7.4 and 6, comparable release

profiles could be observed. As expected, an initial burst release of ferrous from alginate nanoparticles in both buffer solutions (pH 7.4 and 6) can be observed during 7-8 h, accounting for about 25-30% of ferrous from the total encapsulated amount. The initial burst release of ferrous may be due to the rapid hydration of nanoparticles considering the hydrophilic nature of alginate. Then, it followed a more gradual and sustained release phase for the next 78 h. The total amount of ferrous released from alginate nanoparticles at pH 7.4 buffer solution was around 65% after 96 h while it was around 70% at pH 6. The release profile obtained at pH 2 was quite different from those obtained at pH 7.4 and 6. Here, the initial release was low and after 96 h the total release was less than 20%. Higher percentage of ferrous release at pH 7.4 and 6 is a very favorable condition for iron absorption since iron absorption predominantly occurs in the duodenum where the pH is around 6.

Drug release kinetic studies were carried out using model dependent methods which describes the drug dissolution profiles and the best model fitting the release behavior of ferrous from alginate nanoparticles at different pH solutions was selected. According to their adjusted R^2 values (Table 2), the drug release from mucoadhesive alginate nanoparticles in all three different buffer solutions followed the zero order model for the burst release phase during the first 8 h. Thus, it explains the drug dissolution having similar initial release patterns at the pH values tested.

Both Higuchi and Korsmeyer-Peppas models fit with the release behavior of the sustained release phase in all three buffer solutions during later 96 h with high R^2 values. Higuchi model describes the drug release as liquid penetration followed by drug diffusion into the exterior solution depending on the concentration gradient (Singhvi & Singh, 2011). But Higuchi equation is not directly applicable to complex systems such as polymer matrices loaded with drugs which could be subjected to swelling and eroding (Grassi, et al., 2011) whereas Korsmeyer-Peppas model proposed a semi-empirical model in which the drug release is proportional to the sum of two different powers of time which account for the pure diffusivity contribution (Grassi, et al., 2011)(Korsmeyer, et al., 1983). Hence, Korsmeyer-Peppas model best fit with these release studies at pH 7.4, 6 and 2 with high R^2 values: 0.9659, 0.9457 and 0.9613 respectively. Further, the sustained release at pH 7.4 and 6 can be suggested as non-Fickian anomalous diffusion processes with the critical value of n being 0.5 and 0.45 respectively. While, the release behavior at pH 2 ($n = 0.35$) can be best described as a Fickian diffusion in which the release was mainly caused by diffusion only. For spherical particles, $n \leq 0.43$ indicates a Fickian diffusion, while $0.43 < n <$

0.85, indicate a non-Fickian release, an anomalous behavior corresponding to polymer hydration, solvent penetration, drug dissolution and polymer erosion which determine the drug release from hydrophilic polymer materials (Keawchaoon & Yoksan, 2011), (Motwani, et al., 2008)

Table 2: Adjusted R^2 values of curve fitting for five different drug release models obtained for the *in vitro* ferrous release from ferrous loaded alginate nanoparticles at different pH buffer solutions.

Model	R^2 value for burst release phase			R^2 value for sustained release phase		
	pH 7.4	pH 6	pH 2	pH 7.4	pH 6	pH 2
Zero order	0.9960	0.9356	0.9600	0.8965	0.9946	0.9215
First Order	0.9754	0.9189	0.9700	0.9491	0.9447	0.9485
Higuchi	0.9725	0.8848	0.9824	0.9594	0.9615	0.9661
Hixson-Crowell	0.9968	0.91176	0.9533	0.9356	0.9768	0.9205
Korsmeyer-Peppas	0.9877	0.9078	0.9972	0.9659	0.9457	0.9613

Release of 65-70% ferrous in the intestinal pH of 6 and 7.4 is good for oral delivery formulations. According to Goswami et al, the release of insulin from alginate nanoparticles at pH 1.2 was less than 20% while it was 90% at pH 7.4 because at low gastric pH alginate forms a compact acid-gel structure restricting the release of drug from the matrix and also protecting the drug from harsh environmental conditions (Goswami, et al., 2014). Another important factor is that the pK_a of alginate lies well above 3.4, hence in very highly acidic medium, alginate remains undissociated protecting the encapsulated drug. Further, it is known that the gastric juice medium does not breakdown alginate nanoparticles thus providing the stability to the nanoparticles which makes them a suitable carrier in oral formulations (Goswami, et al., 2014). This claim is supported by our observations. Further, the mucoadhesive nanoparticles have the ability to penetrate mucous layer and prolong the residence time and released encapsulant can interact deeply and permeate the intestinal barrier to the bloodstream. This claim was supported by the work done by Sarmento et

al., on insulin loaded dextran sulphate/chitosan nanoparticles. They have observed the hypoglycemic effect for more than 24 h suggesting the insulin internalization, probably through vesicular structures in enterocytes and insulin-loaded nanoparticle uptake through intestine (Sarmento, et al., 2007). Smaller Particle size is also a necessity to be orally absorbed through the intestinal mucosa followed by their passage to systemic circulation (Reis, et al., 2007). In our study, we were able to prepare alginate nanoparticles with sizes around 20-25 nm which may ensure the particle uptake through the intestine. This claim is further supported by the finding of Jani and co-workers who did a study on nanoparticle uptake by the rat gastrointestinal mucosa using different sized particles. They have shown the successful uptake of particles less than 50 nm by small intestine (Jani, et al., 1990). This ensures the improved bioavailability with minimized adverse effects of ferrous loaded alginate nanoparticles.

4. Conclusion

We demonstrate, the preparation of ferrous loaded alginate nanoparticles and its potential as a promising system for oral iron delivery. Alginate nanoparticle preparation was optimized for the initial iron content and the optimized formulation in which the iron concentration was 1%, w/w alginate, yielded nanoparticles in the size range of 15-30 nm with a negative surface charge and good iron encapsulation (75%). FTIR and TGA studies proved the successful loading of iron into the alginate nanoparticles while the EELS study confirmed the loading of ferrous in the presence of ascorbic acid. 70% release of ferrous at pH 6 and less than 20% release at pH 2 are favourable findings for further development of this as an oral iron delivery system. Ferrous loaded alginate nanoparticles thus provide an attractive delivery system for conventional oral iron therapy.

Acknowledgment

This research was financially supported by the HETC QIG Win 3 grant, University of Peradeniya, Peradeniya, Sri Lanka.

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***Int J Pharm, 513*(1-2), 404-409**. doi:10.1016/j.ijpharm.2016.09.053